Declaration of Dr. Marc Jacquemin under 37 CFR § 1.132

1. I, Marc Jacquemin declare and state as follows. I am a professor at the University of Leuven and I am an expert in the field of vascular biology. I am one of the inventors of the U.S. Patent Application No. 10/030,522 entitled 'Ligands for use in therapeutic compositions for the treatment of hemostasis disorders'.
2. I understand that the Examiner has questioned, in the Office Action mailed on May 20, 2004, the fact that single chain variable parts of antibodies directed to the C1 domain of FVIII would be capable of binding FVIII and inhibiting FVIII activity.
3. I herewith present additional data which demonstrate that the single chain variable part of the antibody produced by the cell line Krix-1, which is directed to the C1 domain of FVIII, is capable of inhibiting FVIII activity.
4. In our laboratory, an scFv of KRIX-1 was constructed by adding a linker sequence between the 3' end of the KRIX-1 light chain variable part (VL) and the 5'end of the heavy chain variable part (VH), using standard technology known to the skilled person at the filing date of the present application. The technical details are provided herewith. The resulting scFv-KRIX-1VLVH was cloned into the pPICZαC expression vector and the final pPICZαC-scFv-KRIX-1VLVH(His) vector was used to transform X33 cells for scFv production. The supernatant was tested to demonstrate the presence of a functional scFv fragment. The scFv fragment was purified and after concentration, the scFvKRIX-1VLVH(His) was tested in a FVIII chromogenic assay to evaluate the ability of the scFvKRIX-1VLVH(His) to inhibit FVIII activity. The FVIII inhibitory capacity was evaluated in a Besthesda assay (described in the application as filed): one volume of buffer with scFvKRIX-1VLVH(His) at various concentrations was mixed with one volume of a pool of normal human plasma and incubated for 2h at 37°C. The residual FVIII activity was then measured in a chromogenic assay.
5. The results, presented in Figure 1 enclosed herewith, clearly demonstrate that scFv of the Krix-1 antibody has inhibitory activity on FVIII.
6. I hereby declare that all statements made herein are true and are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonement, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Date: _____

By: Dr. Marc Jacquemin

Figure 1.

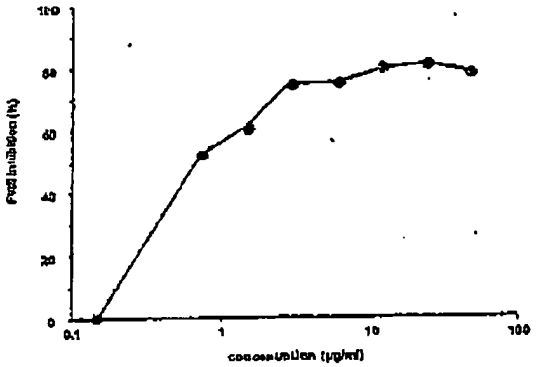

Figure 1: Graph of experimental results showing the FVIII inhibitory activity of scFv fragment of KRIX-1 (scFv-KRIX-1VLVH(His)) produced in Pichia pastoris, in accordance with an embodiment of the invention.

Technical details of the production and characterization of KRIX-1 scFv fragment

1. Cloning of scFv-KRIX-1VLVH in Pichia expression vector

An scFv of KRIX-1 was constructed by adding a linker sequence between the 3' end of the KRIX-1 light chain variable part (VL) and the 5'end of the heavy chain variable part (VH). This was obtained by PCR amplification of KRIX-1 light chain and heavy chains using the following primers:

a) light chain
forward primer:

5'-gtatctctcgag***aaaaga***GAAATTGTGTTGACGCAGTCTCCAGGC-3'

This primer corresponds to the 5' end of the KRIX-1 VL sequence (capital), and contains a *Xho*I restriction site (underlined) and a KEX1 sequence (bold italic);

reverse primer:

5'-*cgccagagccaccicegcctgaaccgcctccacc*TCGTTTGATCTCCACCTTGGTC

This primer corresponds to the 3' end of the KRIX-1 Jk sequence (capital), and contains a part of the linker sequence (italic).

b) heavy chain
forward primer:

5'-*caggcggaggtggctctggcggtggcggatcg*CAGGTMCAGCTGGTGCAGTCTGGG-3'

This primer corresponds to the 5' end of the KRIX-1 VH sequence (capital), and contains a part of the linker sequence (italic);

reverse primer:

5'-gatctctagaTGAGGAGACGGTGACCAGGGTTCC

This primer corresponds to the 3' end of the KRIX-1 JH sequence (capital), and contains a *Xba*I restriction site (underlined).

The PCR products were annealed and a second PCR was performed using the forward primer for the light chain and the reverse primer for the heavy chain. The resulting scFv-KRIX-1VLVH was cloned into the pPICZαC expression vector (Invitrogen, Merelbeke, Belgium)

2. Cloning of scFv-KRIX-1VLVH with His(6)tag in Pichia expression vector

A *SalI* restriction site was added to the scFv-KRIX-1VLVH sequence in order to clone it in frame with the *His*(6) sequence included in the pPICZαC expression vector (Invitrogen; Merelbeke; Belgium). This was obtained by PCR using the forward primer 5'-gtatctctcgag*aaaaga*GAAATTGTGTTGACGCAGTCTCCAGGC-3' corresponding to the 5' end of the KRIX-1 VL sequence (capital), and containing a *XhoI* restriction site (underlined) and a KEX1 sequence (bold italic); and the reverse primer 5'-batggtgacTGAGGAGACGGTGACCAGGGTTCCCCGGCC-3' corresponding to the 3' end of the KRIX-1 heavy chain JH sequence (capital), and containing a *SalI* restriction site (underlined).

The final pPICZαC-scFv-KRIX-1VLVH(His) vector was used to transform X33 cells for scFv production. The supernatant was tested to demonstrate the presence of a functional scFv fragment.

The scFv fragment was purified using the HisTrap Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). After concentration the scFvKRIX-1VLVH(His) was tested in a FVIII chromogenic assay to evaluate the ability of the scFvKRIX-1VLVH(His) to inhibit FVIII activity. The FVIII inhibitory capacity was evaluated in a Besthesda assay.

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Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure

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To : Name of the depositor : JACQUEMIN MARC

Address : Center for Molecular and Vascular Biology
Onderwijs & Navorsing
Herestraat 49
3000 Leuven

I. Identification of the microorganism:

I.1 Identification reference given by the depositor:

KRIX 1

I.2 Accession number given by the International Depositary Authority:

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To : Name of the depositor : JACQUEMIN MARC

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